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COMPARATIVE STUDY OF

MOLECULAR MECHANISMS OF SKIN

IRRITATION AFTER ACUTE

EXPOSURE TO m-XYLENE IN RATS

AND GUINEA PIGS

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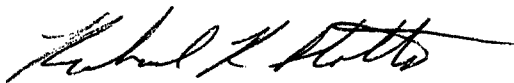
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The animal use described in this study was conducted in accordance with the principles stated in the "Guide for the Care and Use of Laboratory Animals", National Research Council, 1996, and the Animal Welfare Act of 1966, as amended.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE DIRECTOR

RICHARD R. STOTTS, Ph.D.
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13. ABSTRACT (Maximum 200 words)

Organic solvents like xylene are recognized as skin irritants after dermal exposure. The molecular responses to organic solvents that result in acute irritation are not understood. In the present study, we compared and quantified the molecular responses of rat and guinea pigs skin to xylene irritation, since these species differ in their response to other chemicals. We also determined which animal model was more appropriate for predicting xylene-induced skin irritation. Animals were exposed to m-xylene (250 ul) on their shaved back for 1hr using Hill Top Chambers. Zero, one, three and five hrs after the exposure, treated and sham treated skin samples (1g) were collected, homogenized with Tris buffer and measured for early markers of skin irritation. Western blot analysis revealed that IL-1 alpha protein levels increased 3-fold more in rats than in guinea pigs within one hr after xylene exposure. In contract, iNOS protein induction was four-fold greater in guinea pigs that in rats. In rats, the changes in iNOS levels were comparable to the changes in IL-1 alpha levels but occurred two hours later. NO levels, determined by Griess reagent, were elevated four-fold within two hours after the beginning of the xylene exposure in rats. However, in guinea pigs, only a slight change of NO level was observed. Immunohistochemical staining of skin sections using specific antibodies showed immunopositive cells for IL-1 alpha and iNOS. Both antibodies were more predominant in the epidermis of guinea pigs than rats. In addition, oxidant species formation (detected using DCF-DA) was increased over the controls by xylene exposure after 1 hour. As with oxidative species and other early molecular events, histology sections in the guinea pigs revealed more damage and cellular infiltration compared to rats.

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INTRODUCTION

Occupational exposure to chemicals is a source of health concern. One of the responsibilities of the National Institute of Occupational and Safety Health (NIOSH) is the development and demonstration of methodology for assessing human exposure to occupational chemicals. An understanding of the mechanism of this toxic chemical is needed for appropriate risk assessment. Assessment of skin irritation is an important component in estimating health risk for individuals exposed to chemicals. It is considered as one of the major National Occupational Research Agenda Research areas in the United States.

Chemical Induced Skin Irritation

Dermal exposure to chemical substances can lead to a wide variety of skin reactions, such as irritant contact dermatitis, sensitization, altered pigmentation, acne and cancer (Von Burg, 1982; Marks and Kingston, 1985; McCartney, 1996). Contact dermatitis is responsible for major loss of working hours. A main cause of skin inflammation is exposure to irritants (Wahlberg, 1996). The irritant properties of substances are usually tested in animals with the aim of assessing their potential risk to humans. The ability to predict skin irritation or sensitization of organic chemicals remains a key element in assessing the safety of chemicals. A chemical must be absorbed into the viable layers of the skin and cause cellular damage in order to trigger the irritant mechanisms.

Traditionally, testing of skin irritation is conducted in animals (Organization for Economic Cooperation and Development, OECD, 1993). The Draize skin irritation test, in occluded application of test chemicals to skin for 24 hrs, is the basic procedure for classification of skin irritation hazards to man (European Community, 1992). However, assessing the systemic toxicity of chemicals does not predict skin irritation and inflammation (Code of Federal Regulations 1991), we wanted to quantify this effect through detection of early molecular biological events that occur in the skin after exposure to chemical irritants.

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The first change seen in the skin after chemical exposure is erythema and slight edema, which result from cellular damage (Marks and Kingston, 1985). Irritant and sensitizing agents perturb the skin membrane integrity and trigger a cutaneous response by inducing cellular production and release of pro-inflammatory cytokines. These cytokines are considered as critical signals in the cascade of events leading to skin irritation and inflammation (Barker et al., 1991). Our aim is to detect and quantify the time course of early molecular biological events that occur in skin irritation. Although rats and guinea pigs are preferred animals for testing skin irritancy, considerable variations in irritancy response between animal species has been reported (Davies et al., 1972; McDougal et al., 1997). In rats and guinea pigs, organic chemicals like 1,2-dichlorobenzene and chloropentafluorobenzene caused damage to the skin after exposure. 1,2-dichlorobenzene showed histological effects earlier than chloropentafluorobenzene in both species. But the temporal response and severity of the lesions in guinea pigs to these chemicals were greater when compared to rats (McDougal et al., 1997). Here, we presented further studies of species differences in response to xylene. The aim of this study was to compare the molecular responses in rat and guinea pig skin to xylene-induced irritation, and to determine which animal model was appropriate for predicting skin irritation. In addition, the evaluation and characterization of irritant responses have been performed by a visual scoring system to explore dermal irritation and epidermal changes.

Xylene and Skin Irritation

Xylenes are found in number of consumer products, including solvents, paints, plastic, synthetic fibres and gasoline (ATSDR, 1993). m-Xylene as an organic solvent is recognized as a skin irritant following dermal exposure (Rodt et al., 1990). Occupational workers are considered as major populations at risk for xylene exposure. Xylene acts in the viable epidermis and dermis, causing skin damage and edema (Rodt et al., 1990; Elsner, 1994). The mechanism leading to skin irritation and inflammation is not well understood. Over the last decade only a few quantitative studies on irritancy are available for a limited number of compounds (Tupker et al., 1997; Frosch et al., 1994). This literature provides evidence that keratinocytes, which make up the majority of the

epidermis, participate in the immunologic and inflammatory reactions by producing cytokines, chemokines, tumor necrosis factors, etc., (Sauder, 1990). These factors play a significant role in facilitating the induction and elicitation phases of immune responses (Baadsgaard and Way, 1991). Xylene absorption through skin contact may exceed the dose obtained from inhalation in occupational workers (Daniell, et al., 1992). The dermal absorption of m-xylene through the dermis contributes 1.8% of the body burden (Loizou, et al., 1999). Our investigations of dermal irritancy with this solvent should provide necessary information to more accurately predict skin irritation and health risks.

Purpose of this Study

The purpose of this study was to compare and quantify the molecular and histological response in the skin of rats and guinea pigs and to understand the temporal response of the mechanisms of damage after contact with xylene.

MATERIALS AND METHODS

Laboratory Animals and Exposures

Rat and guinea pigs were selected for this investigation because of our experience with these species in dermal absorption studies (both *in vitro* and *in vivo*) in this laboratory (AFRL/HEST). Twenty-five male Fischer rats (CDF® (F-344) /CrlBR, Charles River Laboratories, Raleigh, NC) and twenty-five male Hartley guinea pigs weighing between 250 and 350 grams were used (5 animals per time point). Initial serology and pathology evaluations indicated the animals were healthy and free of diseases. The animals used in this study were handled in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals (NRC, National Academy Press, 1996). The rats and guinea pigs were housed one per cage in plastic shoebox cages and provided a 12-hr light/dark cycle. Temperature was maintained between 18° and 26°C, and relative humidity was maintained between 30 and 70%. Food (Formulab Rodent Diet, PMI Feeds, Inc., St. Louis, MO) and water were provided *ad libitum*. On the study day, rats

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and guinea pigs were anesthetized with isoflurane (1-chloro 2,2,2-trifluoroethyl difluoromethyl ether) using a vaporizer (Ohio Medical Products). The fur on the back of the animal was closely clipped with Oster® animal clippers (McMinnville, TN) and a number 40 blade, while taking care not to damage the skin. An Oster® finishing clipper (0.22mm) was used to carefully remove the remaining fur stubble (Jepson and McDougal, 1997). A template with size equivalent to the diameter of the outside edge of the Hill Top chamber was used to mark 4 to 5 circles with a waterproof marker near the dorsal midline. A Hill Top chamber containing 0.250 ml of m-xylene (99% purity, purchased from Eastman Kodak Company, Rochester, NY) was placed on each skin site and the chamber was secured with elastic adhesive bandage (Elastoplast® Belesodrof, Inc., Norwalk, CT). The animal was placed back in the shoebox cage (one in a cage) and allowed to recover from anesthesia.

Skin Isolation and Preparation

After one hour, the Hill Top Chamber was removed and excess solution on the exposed area was wiped with cotton gauze. Zero, one, two, four, and six hours after the beginning of the exposure, animals were euthanized with CO_2. Five skin samples were collected from under the exposure chambers. One skin sample was fixed with 10% neutral buffered formalin, routinely processed into paraffin embedded sections and prepared for histological or immunohistochemical staining. Two of the remaining skin samples were processed for mRNA analysis, and two samples were used for protein and NO analysis. The skin samples that were processed for mRNA alone were immediately placed into 10 mM vanadium ribonucleoside solution (Life Technologies, Rockville, MD) for 30-60 seconds. All the samples were removed and placed between two clean metal combs, held together with locking pliers. A scalpel was then passed between the teeth of the comb to cut the skin into strips. The skin was rotated 90° and cut again into small squares. Immediately after mincing, the skin pieces were placed onto a piece of aluminum foil and flash-frozen in liquid nitrogen.

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Western Blot Analysis for IL-1α and iNOS Level

Proteins (50 μg/lane) from skin samples were denatured at 90°C for 5 min and were resolved on 8% and 12% SDS-PAGE for iNOS and IL-1α respectively, according to the procedure of Laemmalli (1970). Following separation, the proteins were transferred to a nitrocellulose membrane according to the method of Towbin et al (1979). The membrane was then blocked with 5% non-fat dairy milk and probed overnight at 4°C with either anti-iNOS or anti-IL-1α polyclonal antibodies (1:1000; Affinity Bioreagents, Inc. Golden, CO). Following washout with phosphate-buffered saline (PBS), membranes were incubated with peroxidase conjugated secondary antibody (1:2000) for 1 hour and washed three times with PBS. The IL-1α and iNOS bands were visualized using enhanced chemiluminescence and autoradiography. The levels of the IL-1α and iNOS were quantified by comparing band intensities and expressed as a percent change from 0 hour for each group (mean ± SEM).

RNA Isolation and RT-PCR

Frozen skin samples (~125 mg) from each time period (n=10) were pulverized in a Bessman stainless steel pulverizer (Fisher Scientific) cooled with liquid nitrogen. Pulverized samples were placed in 1.0 ml of TriReagent, homogenized using a Tissue Tearor™ homogenizer (Biospec Products, Inc., Bartlesville, OK), and total RNA was isolated according to the manufacturer's instructions. One μg of total RNA from each sample was reverse transcribed into cDNA using the AMV first strand cDNA synthesis kit (Boehringer Mannheim) according to the manufacturer's instructions. The 20 μl reaction mixture contained 1X reaction buffer, 5 mM $MgCl_2$, 10 mM dNTP, 0.08 μg p(dN)$_6$ primers, 50 U RNase inhibitor, and 20 U AMV reverse transcriptase was incubated at 25°C for 10 min, 42°C for 60 min, 95°C for 5 min, and then quickly placed on ice. For iNOS, the 100 μl PCR reaction mixture consisted of 1X reaction buffer, 2.0mM $MgCl_2$, 25nM dNTPs, 100nM of each primer (5'GGIGGITG(C/T)CCIGCIGA(C/T)TGG3' and 5'TCICCATTICCAAAIGTI(C/G)(A/T)IGTNACNAC3'), 10 μl of cDNA template, and

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U Taq polymerase (Promega). The reaction mixture was initially denatured for 2 min at 94°C, then followed by amplification for 35 cycles having denaturing (94°C), annealing (57°C), and extension (72°C) steps lasting 45 sec, 45 sec, and 2 min, respectively. A final extension step at 72°C lasted for 5 min. For IL-1α, PCR was performed as previously described (Hurley et al. 1999).

Standard curve preparation

A 428 bp region of the iNOS gene was amplified by PCR from a cDNA fragment kindly provided by Dr. Kenneth Hensley (Oklahoma Medical Research Foundation). A 589 bp region of the IL-1α gene was amplified by PCR from the America Type Culture Collection mouse IL-1α clone. The PCR products were electrophoresed on 1% agarose gels and visualized following ethidium bromide staining. A band corresponding to the amplified fragment (IL-1α or iNOS) was cut out of the gel, and the DNA was purified using the agarose gel DNA extraction kit (Boehringer Mannheim). Insertion of the purified DNA into the pCR TOPO II TA cloning vector, transformation of electrocompetent *E. coli*, and selection and screening of clones was done according to the manufacturer's instructions (Clontech). Plasmid DNA was isolated from liquid cultures of selected clones using the Wizard Mid-Scale Plasmid prep kit (Promega). The presence of the PCR product in each clone was determined by restriction enzyme digestion and PCR.

Purified pCR TOPO II vectors containing either the IL-1α or iNOS PCR fragment were linearized following restriction digestion for 1 hr at 37°C with EcoRV or HindIII. From this template, RNA was synthesized in vitro using the MaxiScript kit (Ambion, Austin, TX), quantitated spectraphotometrically, and aliquoted into concentrations corresponding to 0.5, 5.0, 50, 500, 5000, and 50,000 fg RNA/μl for iNOS and IL-1α. One μl of each dilution was reverse transcribed into cDNA using the AMV first strand cDNA synthesis kit (Boehringer Mannheim) according to the manufacturer's instructions. The 20 μl reaction mixture contained 1X reaction buffer, 5 mM MgCl$_2$, 10 mM dNTP, 0.08 μg p(dN)$_6$ primers, 50 U Rnase inhibitor, and 20 U AMV reverse transcriptase. The reaction

was incubated at 25°C for 10 min, 42°C for 60 min, 95°C for 5 min, then quickly placed on ice. Ten μl of the reaction mixture was used for PCR of iNOS or IL-1α as described above.

Quantitation of PCR was performed using a fluorescent microplate assay (Romppanen et al., 2000). In a Costar 96-well microplate, 10 μl of each PCR reaction (standard and samples) was combined with 90 μl Tris-EDTA (TE) buffer, and 100 μl of the dsDNA-binding dye PicoGreen® (Molecular Probes, Eugene, OR). The mixtures in each plate were incubated in the dark for 5 min at room temperature, and analyzed using a Cytofluor 2300 fluorescent microplate reader (Millipore, Bedford, MA) with excitation and emission spectra of 485 nm and 530 nm, respectively. The fluorescence value of each sample was plotted vs. Log concentration of the standard curve. Data were expressed as mean fg mRNA/μg total RNA.

Determination of Nitric Oxide Production

Determination of nitric oxide production was achieved by indirectly measuring nitrite accumulation in the supernatant solution. Nitrite (NO_2^-) and nitrate NO_3^- are stable products of NO. Nitrite was determined by mixing 100 μl of supernatant and 100 μl Griess reagent (Klebanoff, 1993). The reaction was allowed to proceed for 15 min at room temperature (RT) and the absorbance at 550nm measured by spectrophotometer. The concentration of NO_2^- was calculated from a pre-calibrated standard curve using sodium nitrite ($NaNO_2$) as standard.

Nitric Oxide Synthase Activity

Nitric oxide synthase (NOS) activity was measured from xylene exposed rat and guinea pig skin according to the method described by Bredt (1999). Skin samples were homogenized in five volumes of buffer containing 0.25 M sucrose, 20 mM Tris (pH 8.0), 1 mM EDTA and 1 mM dithiothreitol. The homogenate was centifuged at 15,000 g for 30 min at 4°C and the resulting supernatant served as enzyme source. NOS activity was determined by the conversion of [^{3}H-L-arginine] to [^{3}H-L-citrulline]. The standard

reaction mixture contained (total volume 0.1 ml): 50 mM Tris.HCl pH (7.6), 2 mM CaCl$_2$, 100 mM NADPH, 100 mM ^{3}H-L-arginine (approximately 20,000 dpm, and specific activity of 64.2 Ci/mmol) and 100 μg of the enzyme source. The reaction was carried out at 37^{0}C for 10 min. and the reaction was stopped by the addition of 400 ml of stop mix containing 20 mM sodium acetate, pH 5.2, 2 mM EDTA, 2 mM EGTA and 1 mM L-citrulline. The ^{3}H-L-citrulline formed in the reaction mixture was separated using Dowex AG W-X8 resin and the radioactivity was measured using Perkin-Elmer liquid scintillation counter. The protein content was determined by Bicinchoninic acid (BCA) reagent kit from Pierce.

Detection of Intracellular Oxidative Species

Oxidative species were determined from skin of both rat and guinea pigs following xylene exposure according to the method described earlier (Royall and Ischiropoulos, 1993). Oxidative species generation was assessed by using cell permeable non-fluorogenic probe, 2,7-dichlorofluorescin diacetate (DCF-DA). In the presence of oxidants, DCF-DA is oxidized into fluorescent 2,7-dichlorofluorescein (DCF). Tissue homogenates (40 μg protein, in a volume of 200 μl) were incubated with 5 μl of DCF-DA (10 μM) for 30 min at 37°C. The fluorescent product DCF was quantified by a spectrofluorometer at the excitation 485 and emission 530 nm. Changes in fluorescence were expressed as percent of control.

Detection of Lipid Peroxidation

As a direct measure of the ability of the xylene to induce oxidative stress, thiobarbituric acid reactive substances (TBARS), an index of lipid peroxidation was quantified in skin homogenates of control and xylene exposed samples according to the method of Ohkawa *et al.*, (1979) with minor modifications. 100 μl of SDS, 1.3 ml of 0.5% (w/v) thiobarbituric acid in 20% glacial acetic acid (pH 3.5) were added to 100 mg tissue homogenate. Samples were incubated for at 80°C for 30 min, cooled to room temperature, and centrifuged at 5000 x g for 5 min. TBARS formation was determined by

recording the absorbance at 532 nm and estimated using an extinction coefficient of 1.56 x $10^5 M^{-1}$ cm^1. The changes in absorbance were expressed as percent of control.

Immunohistochemical Staining of IL-1 α and iNOS

Deparaffinized and rehydrated sections were subjected to immunohistochemical staining for IL-1 alpha and iNOS. An avidin-biotin-peroxidase complex method was used according to the procedure included in the Vectastain *elite* ABC peroxidase kit (Vector Laboratory, Burlingame, CA). Briefly, sections were washed with distilled water and incubated with 0.1% Triton X-100 in 0.1% sodium citrate for 30 min at RT prior to quenching of endogenous peroxidase activity with 0.3% H_2O_2. Sections were washed in PBS, and incubated with 5% normal goat serum in PBS for 30 min at RT. Skin sections were incubated (1:100) either with rabbit anti-rat IL-1 alpha or with rabbit anti-rat iNOS for 30 min at room temperature. Following PBS washing, the sections were incubated with secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG (1:500) in PBS for 30 min at RT. Peroxidase was detected by the addition of 0.025% diaminobenzidine tetrahydrochloride (DAB; DAKO Corporation, Carpinteria, CA). Finally, all slides were lightly counterstained with hematoxylin, dehydrated and mounted with Paramount® mounting media. The appropriate controls were included during each run to rule out non-specific staining.

Histopathology

Biopsies were elliptical in shape with the long axis oriented parallel to the direction of hair growth. This provided a longitudinal microscopic view of hair follicle structures rather than undesirable cross sectional perspectives. Skin specimens were flattened on a piece of paperboard or photographic paper and gently stretched before immersion in 10% neutral buffered formalin for 24 hours. Following fixation, skin samples were processed using standard operating procedures for paraffin processing, embedding, and sectioning.

Hematoxylin and eosin stained slides were assessed in random order by group and scored as described previously (McDougal et al., 1997).

RESULTS

CELLULAR ASSESSMENT OF DAMAGE

Il-1α Responses of Rats and Guinea Pigs

The object of this study was to evaluate the species differences in response to their skin exposure to an organic solvent, m-xylene. Rats and guinea pigs skin were processed as described earlier for estimation of IL-1α protein levels. The densitometric quantification of IL-1α, as determined by western blot, is shown in Fig.1. At all time points tested, IL-1α protein levels were increased in both species after xylene exposure relative to respective controls. In rats, 1 hr exposure to this solvent increased IL-1α protein levels markedly from 74 to 156% over the control at various time points compared to guinea
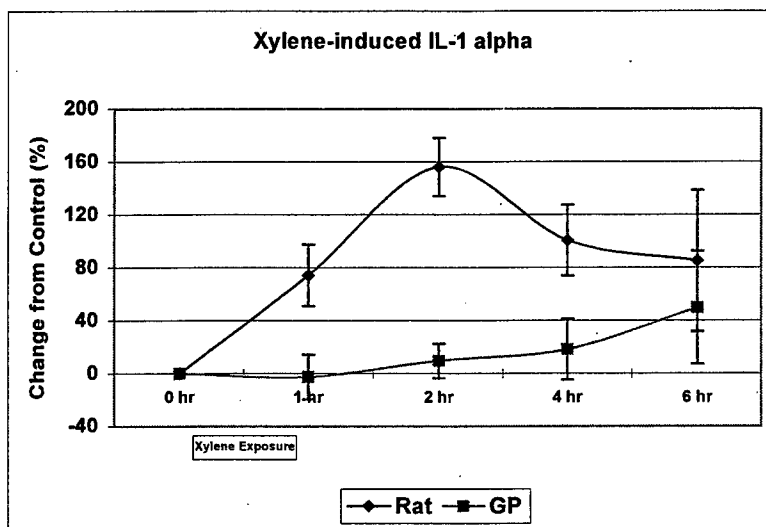

Fig.1. Effects of xylene on IL-1 alpha protein in rat and guinea pig skin. Skin was processed for the measurement of IL-1 alpha using specific IL-1 alpha antibody by Western Blot. Error bars depict standard error of the mean (n=5 at each time point).

pigs which only increase by 10 to 50%. The IL-1α response in rats was about three times larger than guinea pigs. Rat IL-1α levels increased during the exposure and reached maximum at two hours after the beginning of the exposure.

Inducible Nitric Oxide Synthase Responses of Rats and Guinea Pigs:

In skin irritation or inflammation, a large amount of nitric oxide is produced through induction of inducible nitric oxide synthase under the influence of proinflammatory cytokines. After xylene exposure for 1 hr, the profile of iNOS protein levels was investigated by western blot using monoclonal antibody against iNOS. A protein band of 130KD, corresponding to the expected position of iNOS was found in the samples from exposed skin, while the same band was hardly seen in control skin samples. The increased level of iNOS in xylene exposed skin samples was confirmed by immunostaining (Fig.8).

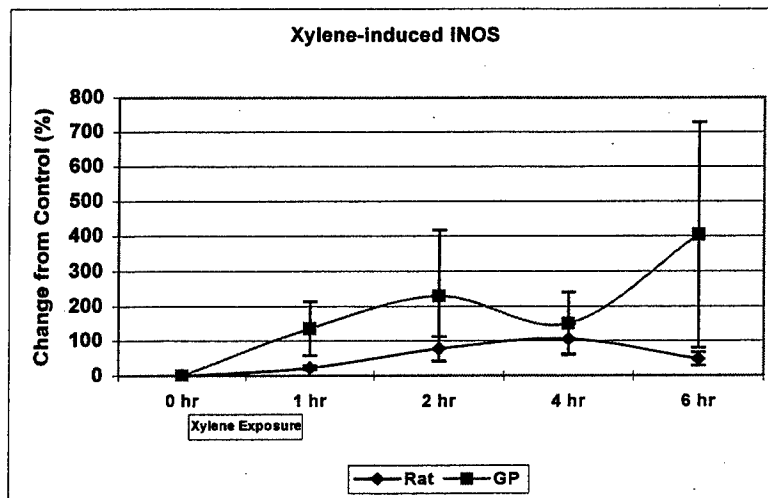**Fig. 2** Effects of xylene on iNOS protein in rat and guinea pig skin. Xylene-exposed skins were processed for the measurement of iNOS using specific iNOS antibody by Western Blot. Error bars depict standard error of the mean (n = 5 at each time point).

Fig.2 shows that the guinea pig iNOS response was about four times greater than the rat response. iNOS levels in the guinea pigs were highest 6 hr after the start of the

exposure. The variability in guinea pig iNOS levels may be due to antibody that lacked specificity. Rat iNOS protein levels were increased at 4 hr after the beginning of the exposure and decreased slowly at the last time point of exposure.

Effects of xylene on Nitric Oxide

Elevated nitrite in the xylene-exposed sample was assayed by Greiss reaction as an indication of NO production. As shown in Fig.3, xylene stimulated nitric oxide production in both species. However, the nitric oxide levels in rats and guinea pigs showed a different response. Like NOS protein levels, the nitric oxide levels (nitrite) in
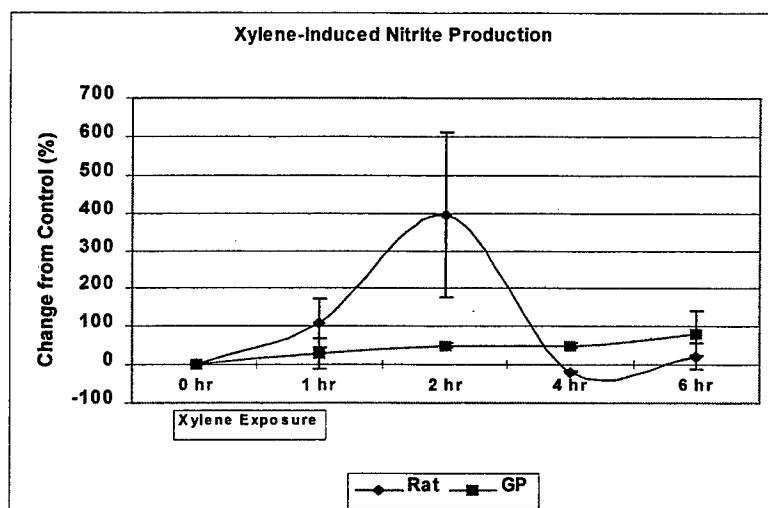
Fig. 3 Effects of xylene on Nitrite production in rat and guinea pig skins. Xylene exposed skin samples from both species were processed for estimation of NO production by measuring accumulated nitrite in the supernatant of the each samples with Greiss reaction. Error bars depict standard error of the mean (n = 5 at each time point).

rats peaked (400%) two hrs after the beginning of the exposure and decreased gradually. However, in guinea pigs nitric oxide levels changed slightly (82%) during the 6-hr period after the beginning of the xylene treatment. Increased nitrite levels indicate the stimulating effect of xylene on nitric oxide production in rat.

Effects of xylene on iNOS m-RNA levels

RT-PCR was performed to examine whether the stimulating effect of xylene on iNOS and NO production in rat and guinea pigs are due to the increased synthesis of iNOS mRNA. Fluorometric analysis of mRNA mass was plotted against Log concentration of the standard curve and the change of iNOS mRNA mass formed from 0 to 6 hr post-exposure to xylene.
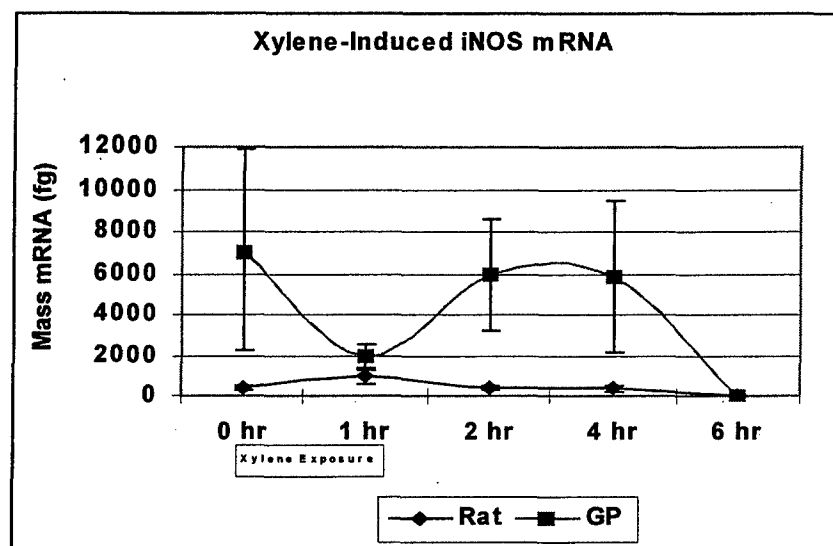

Fig 4. RT-PCR analysis of iNOS mRNA expression in rat and guinea pigs. The changes in the expression (mass) of messenger RNA for iNOS with 1 hour exposure to xylene was expressed as change of iNOS mRNA over 0 hour samples. The value represent mean ± S.E from five separate animals.

As shown in fig. 4, xylene exposure (1 hr) increased rat iNOS mRNA expression during 1 hr post exposure by ~25% above 0 hr samples. The expression of iNOS mRNA steadily decreased through 6 hr. This indicates that induction of iNOS mRNA at 1 hr into the exposure may be sufficient to induce NOS protein synthesis as observed (fig.2). However, the changes in iNOS mRNA in guinea pigs showed more variability among the individuals. In comparison to rats, xylene-induced guinea pig's iNOS mRNA response was stronger, but the change in mRNA levels was not reflected in protein levels (fig.2).

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Effects of Xylene on NOS Activity

L-citrulline assay was performed to measure the total NOS activity in rat and guinea pigs exposed to xylene (1 hr). This radiometric analysis demonstrated increased levels of total NOS activity in rat skin compared to 0 hour sample (Fig.5), but NOS activity differs between rats and guinea pigs. As shown in Fig. 5, rat NOS activity increased at 1hr and then peaked (25 to 30%) at 4 hours over the zero hour samples. The change in NOS activation due to xylene exposure in guinea pigs was slightly variable compared to 0-hour samples. Thus, species difference was seen in xylene-induced NOS activation.
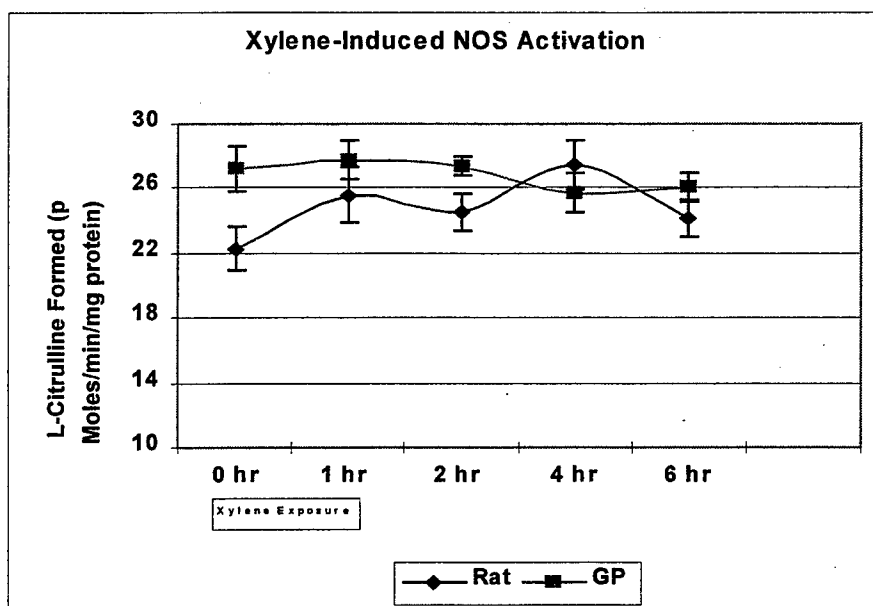
Fig. 5 Effects of xylene on Total NOS activity in rat and guinea pig. Skin samples were homogenized and supernatant served as enzyme source. NOS activity was determined by the conversion of [^{3}H-L-arginine] to [^{3}H-L-citrulline] and expressed as pmoles formed / min /mg protein. The value represent mean $\pm$ S.E from five separate animals.

Xylene-Induced Oxidative Species

Xylene produced an increase in fluorescence upon addition of oxidant dye, DCF-DA to the supernatant fraction of exposed and unexposed samples. Fluorescence reflects the formation of the oxidant species due to the oxidation of dye by oxidative radicals that accumulated in skin following xylene exposure. The intensity and duration of the

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response of these two species was different. As shown in Fig. 6, xylene exposure produced a moderate increase in oxidative species in rat skin by 32%, 2 hr after beginning of exposure and then slightly decreased but remained above the control level. In contrast, the oxidant species significantly increased in guinea pig skin by 62% immediately after 1 hr beginning of exposure, and remained slightly variable during the rest of the time period.
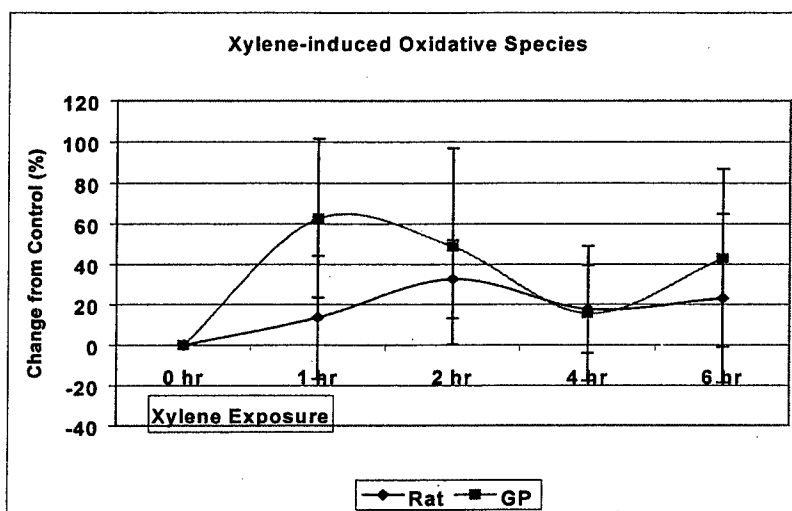

Fig. 6 Xylene-induced oxidative species in rat and guinea pigs skin. Xylene exposed skin samples were homogenized and supernatants were loaded with non-fluorescent dye, DCF-DA. Fluorescence as an indicator of oxidative species formation was measured and compared with unexposed samples. The difference in the fluorescence was converted into % change. Values represent mean $\pm$S.E from 5 sets of animals.

Xylene-Induced Lipid Peroxidation

Further evidence of oxidative process involvement in xylene-exposed skin was obtained by measuring TBARS formation in both rats and guinea pigs. A 4-fold increase of lipid peroxidation occurs in rat skin at 4 hr after the beginning of xylene exposure (Fig. 7). However, in guinea pigs xylene exposure mildly increased (30%) lipid peroxidation over the time period. The changes in the peroxidation of membrane phospholipids in rat indicate the involvement of oxidative stress mediated peroxidation in xylene exposure.

However, in xylene-exposed guinea pigs, the slight increase in peroxidation of membrane phospholipid was noted.
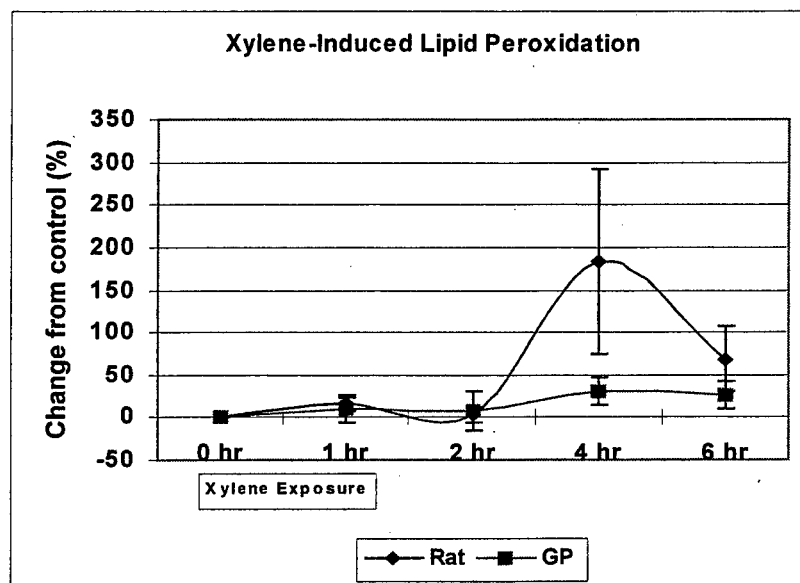

Fig.7 Xylene-induced lipid peroxidation in rat and guinea pig skin. Xylene exposed skin samples were homogenized and supernatants were incubated with thiobarbituric acid at 80 °C for 30 min and TBARS formation (absorbance) was recorded spectrofluorometrically at 532 nm. The % of change in absorbance was recorded as difference between control and exposed samples. Values represent mean ±S.E from 5 sets of animals.

2. HISTOLOGICAL ASSESSMENT OF DAMAGE

Immunohistochemistry of IL-1 α and iNOS

In order to confirm and compare the molecular biomarkers of dermal irritation and inflammatory response (IL-1 alpha and iNOS) to xylene in rats and guinea pigs, skin sections from control and xylene-treated samples (5 hrs after 1 hr xylene exposure) were processed for IL-1 α and iNOS immuno histochemical staining. Identification of immunopositive cells for IL-1 α and iNOS with their respective antibodies revealed more immunopositive cells in xylene-exposed rats and guinea pigs (~ two fold) compared to

16

sham treated controls. Guinea pig epidermis showed more intense staining than rat epidermis. Both IL-1 α (Fig.8) and iNOS (Fig.9) positive staining was predominant in the epidermis rather than dermis. These immunohistochemistry results provide a visual explanation of changes that occur due to xylene exposure and correlate with concurrent molecular data.
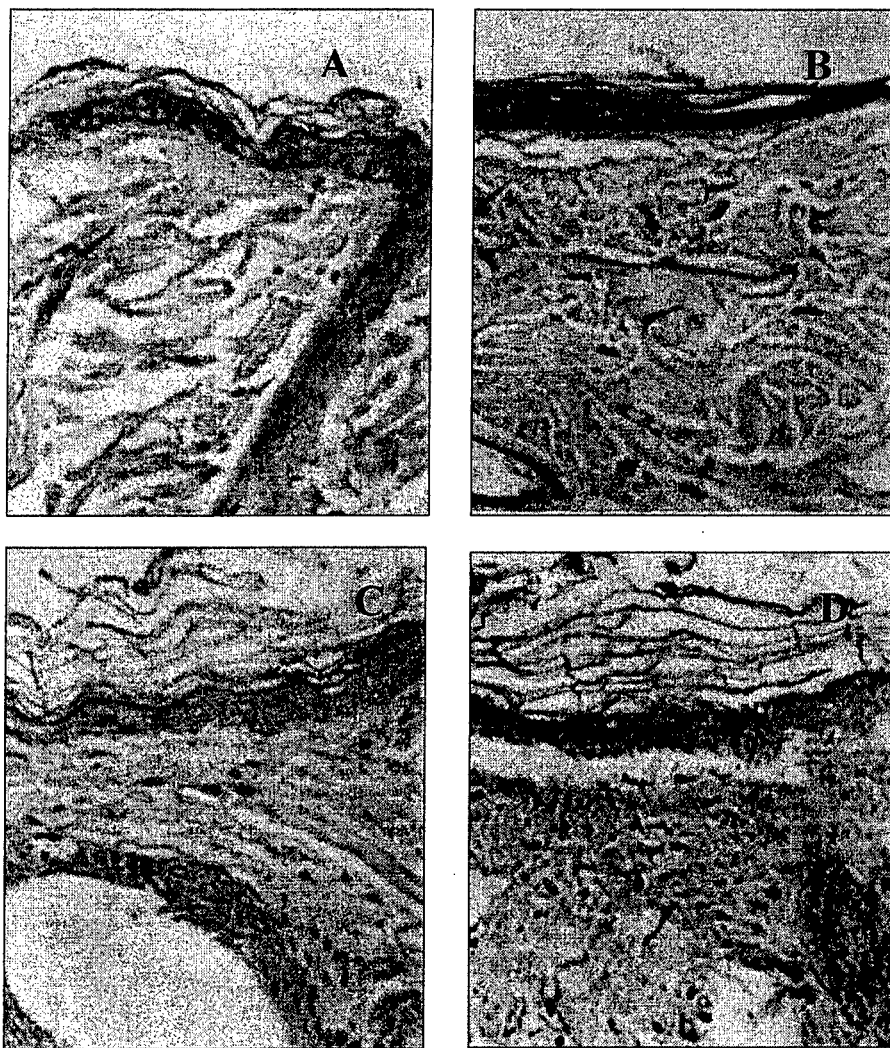
Fig.8. Skin section (X 40) from rat (B) and guinea pig (D) exposed to m-xylene for one hour shows higher immunohistochemical staining of interleukin-1 alpha in the epidermis compared to 0 hr samples (A and C, respectively). Skin sections are taken 5 hours after the end of exposure.
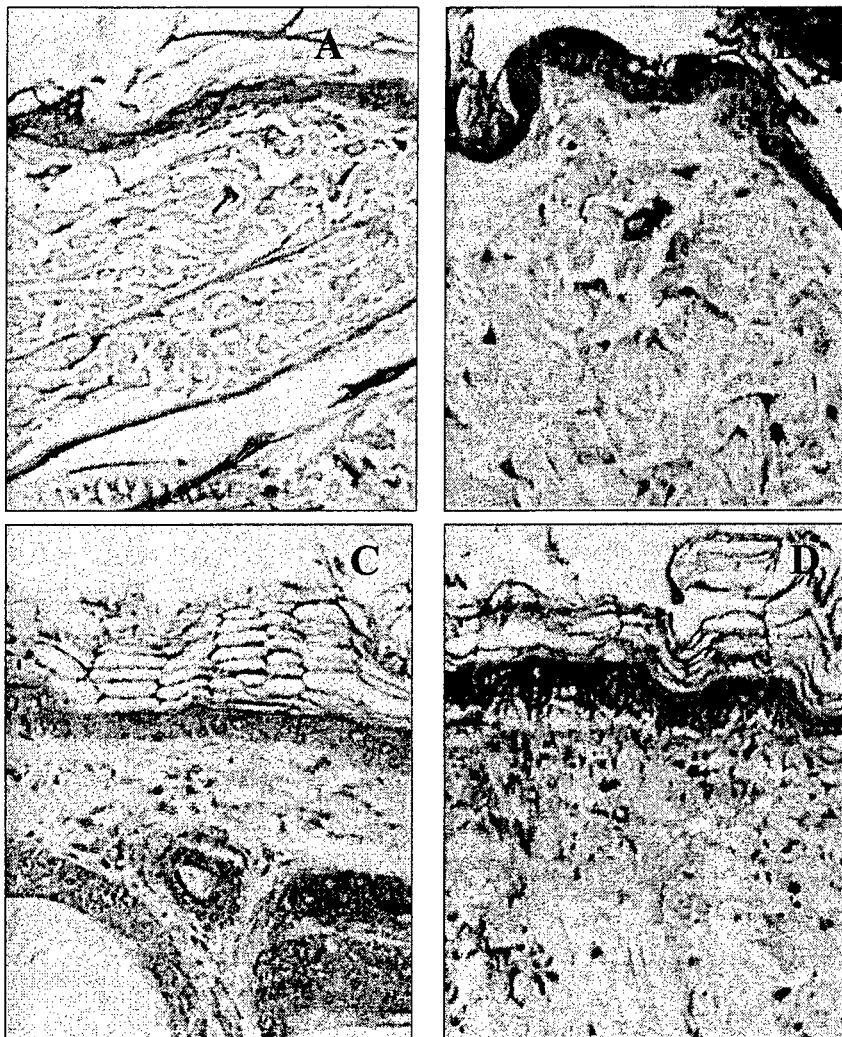
Fig. 9 Skin section (X 40) from rat (B) and guinea pig (D) exposed to m-xylene for one hour shows higher immunohistochemical staining of inducible NOS in the epidermis compared to 0 hr samples (A and C, respectively). Skin sections are taken 5 hours after the end of exposure

Histopathology

To compare the time course and severity of the histopathological responses in rats and guinea pigs, histological evaluation of the response to xylene was performed in non-exposed skin and skin five hours after the end of exposure. In our acute dermal exposures with xylene, inflammation was more pronounced in guinea pigs than rats. In

addition, a one- hour xylene exposure caused a response in rats and guinea pigs which was progressively increased at 2, 4 and 6 hours. Skin histopathology also revealed the presence of epidermal separation and cellular infiltration into the dermis in a similar pattern in both species with a significant increase in guinea pigs (Fig. 10). Compared to rats, guinea pigs responded to this irritating solvent with greater severity of epithelial necrosis. However, in rat sections, intraepidermal separation was also present with discontinuous separation at the dermal and epidermal junction.
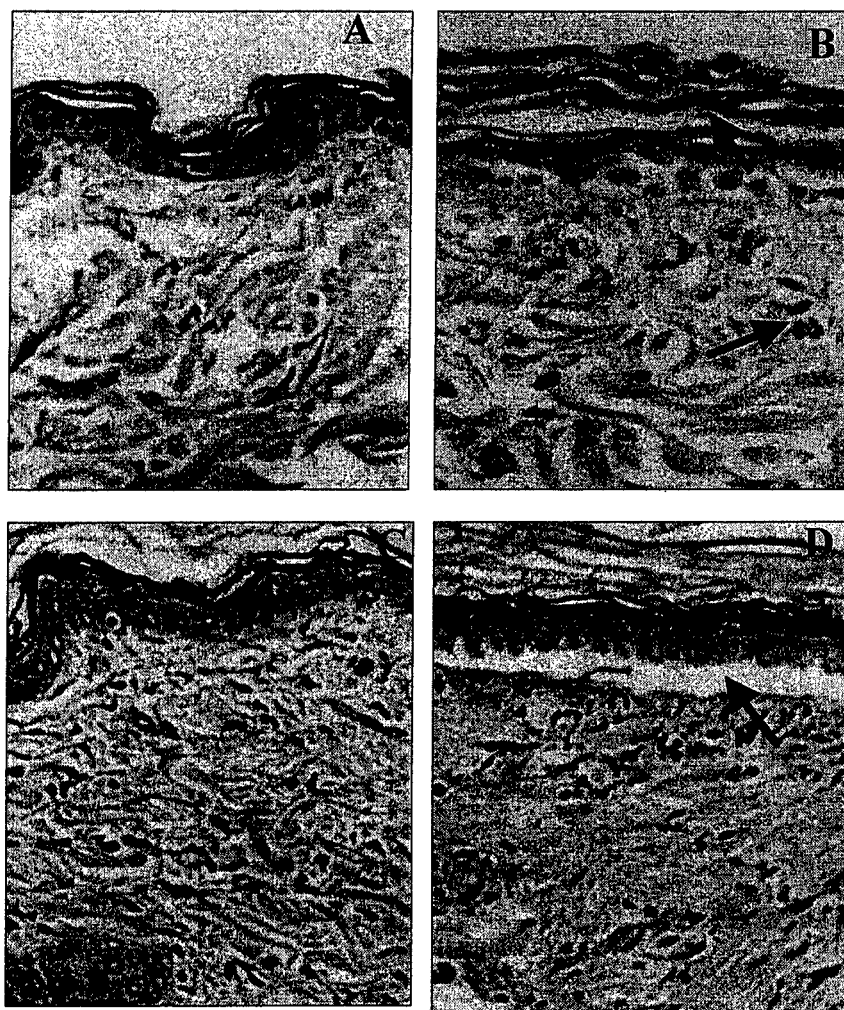

Fig. 10 Skin sections (X 40) treated one hour with m-xylene shows morphological damage in rats (B) and guinea pigs (D) compared to respective control animals (A &C) in the epidermis. Samples taken from control and 5 hours after one 1 hr exposure were compared. Arrows in xylene-exposed skin depict intraepidermal separation. In the dermis, an arrow shows infiltration of granulocytes.

In the guinea pig sections, intraepidermal separation was minimal but there was nearly complete separation of the epidermis from the dermis. In both rats and guinea pigs, cellular infiltration appears localized to the dermal epidermal junction.

Histological Assessment Score

Mean histopathologic assessment scores in rats and guinea pigs are presented in Table 1 below. Increased histopathologic assessment scores were observed from one, two, four and six hours time points. These results indicate that the earliest xylene induced histopathological change, (granulocyte migration), can be seen as early as two hours and changes were most prominent by 6 hours after the start of exposure.

Table 1. Comparative Mean Histopathologic Assessment Scores for one-hour m-Xylene Exposure in Rats and Guinea Pigs

Time (Hours)	Mean Scores* (range)	
	Rat	Guinea Pig
0	0 (0-0)	0 (0-0)
1	1.6 (1-2)	3.3 (3-3.5)
2	1.6 (1-2)	3.7 (3-4)
4	2.0 (2-3)	3.8 (3-4)
6	1.9 (2-3)	4.0 (4)

*Scoring guidelines based on the following criteria:
C (control) = a) essentially normal tissue (<2 granulocytes) or b) the least recognized change from a group of five animals
1 (minimal change) = increase in granulocytes (>2 granulocytes); <5% separation of keratinocytes from basement membrane
2 (mild change) = > 2 granulocytes; prominent segmental basement membrane separation
3 (moderate change) = inflammation; extensive basement membrane separation; epithelial necrosis; <5% epithelial necrosis
4 (severe change) = granulocytes TNTC; extensive basement membrane separation; extensive epithelial necrosis; epithelial transmigration of granulocytes. n=5 at each time point.

DISCUSSION

In this study, we evaluated and compared the molecular and biological responses of the skin in rats and guinea pigs to m-xylene, and determined if either of the animal models was appropriate for predicting skin irritation. The irritant, m-xylene (>99%) was placed on the skin of animals for 1 hr. to represent a realistic occupational exposure to organic solvents. Application of m-xylene for a short time resulted in specific skin damage that correlated with the induction of IL-1α proteins. The increase in protein was greater and earlier in rats than in guinea pigs. The topical treatment with m-xylene caused severity with epithelial necrosis and basement membrane separation in both species over the observed time period. The temporal response and the severity of the lesions induced by xylene differed in rat and guinea pigs. These changes were typically great in guinea pigs compared with rats. Analysis of quantitative histological scores showed that exposure of guinea pig's skin with m-xylene resulted in a significant increase in dermal inflammation with more granulocytes. These data suggest that cytokines, in particular IL-1α and its regulation of iNOS may have a central role in the molecular mechanisms of xylene-induced skin irritation.

Skin irritation results from loss of keratinocyte membrane integrity, which triggers a cutaneous response by releasing cellular pro-inflammatory cytokines. These cytokines are considered as a critical signal in the cascade of events leading to skin irritation and inflammation (Barker, et al., 1991; Ponec and Kempenaar, 1995). Among the number of cytokines, IL-1α is a major interleukin present in keratinocytes (Kupper, et al., 1986) and is associated with skin irritation (Corsini, et al., 1996a). IL-1α, as the primary and pro inflammatory cytokine, upon stimulation further releases IL-1α and other cytokines. In the present study, we have shown that the induction of IL-1α induced by m-xylene occurred earlier during the observed time period. This was consistent with the results obtained from 3,5-xylenol, chloroxylenol, sodium dodecyl sulfate and other chemicals (Corsini et al., 1996a; Corsini and Galli, 1997; Newby et al., 2000). However, the time of induction of this protein varied between rats and guinea pigs. The observed

similar IL-1α release associated with skin irritation following tributyltin (Corsini et al., 1996a) and JP-8 (Kabbur et al., 2000) exposure has been reported. This suggests that a similar immunological event appears to be induced within one hour after xylene exposure. Previous studies with other skin irritants such as sodium lauryl sulphate (SLS) or 1-chloro-2, 4-dinitro benzene (DCNB) induced the expression of several genes, including IL-1α and other cytokines involved in dermal irritation and inflammation (Corsini and Galli, 1997).

As protein levels are regulated by protein synthesis (mRNA controlled), in the present study, the increased IL-1α protein levels in response to xylene exposure may be due to the up-regulation of IL-1α mRNA. In skin, IL-1α gene expression and related protein are shown to be up-regulated following treatment with SLS (Shivji et al., 1994), JP-8 (Kabbur et al., 2000) or other compounds (Kupper, 1990; Schwarz and Luger, 1989). This suggests IL-1α activation occurs by up regulation of IL-1α mRNA within 1 hr after xylene exposure. In addition, the immunopositive expression of IL-1α in xylene exposed rat and guinea pig skin further supports the induction of IL-1α. However, IL-1α immunopositive expression was shown to be greater in guinea pigs than in rats. This reflects the pathological changes observed in the skin in both species. Although we saw differences in the time course of activity, immunopositive staining and skin damage between these two species, this study revealed significant increases in IL-1α levels in both species in response to xylene.

IL-1α can enhance the expression of iNOS and other genes in inflamed skin (Knowles and Moncada, 1994; Ormerod et al., 1997: Frank et al., 1999). Several studies demonstrated iNOS up regulation in human inflammatory diseases and skin disease (Kuhn, et al., 1998, Ormerod, et al., 1997, Bruch Gerharz et al., 1996). In the present study, we examined whether xylene-induced IL-1α could induce iNOS and related protein in both species. The results showed that treatment with xylene stimulated both iNOS mRNA and protein levels. The same phenomenon was seen after treatment of rat skin with JP-8 jet fuel in our laboratory (Kabbur et al., 2000). The degree of stimulation on iNOS mRNA in rats and guinea pig skin was variable. In rats, during IL-1α onset at 1 hour, iNOS mRNA and subsequent protein were up regulated. Whereas in guinea pig,

changes in the expression of iNOS mRNA showed a variable response to xylene. This discrepancy may be due to the variability among the individuals at the transcription level. But in guinea pig, iNOS protein levels were elevated as early as 1 hr. The change in iNOS mRNA levels correlates with change in protein level, indicating that iNOS regulation occurs at the transcriptional level. Moreover, immunolocalization of iNOS in both species particularly in guinea pigs supported this explanation, as it showed strong distribution compared to control animals.

This study demonstrated that IL-1α and iNOS are up-regulated in xylene exposed skin. This may lead to the production of NO to mediate free radical induced cell injury and death (Cotton et al., 1999). In rats, remarkable increase of NO in response to xylene indicates the induction of a functionally active NOS enzyme. As expected, the NOS activity measured by the conversion of [^{3}H]L-arginine into [^{3}H]L-citrulline was significantly higher in xylene treated rats compared to 0 hour samples, with little change in guinea pigs. In rats, xylene stimulated total NOS activity increased at 1 hour and then peaked at 4 hours. Moreover, the production of NO paralleled NOS activation. However, the decreased NO after 2-hour may be due to utilization of NO for peroxinitrite formation. In rats, it is possible that NO formation can react quickly with excess superoxide to form different products such as peroxynitrite, thus reducing NO levels (Bartosz, 1996; Chamulitrat et al., 1993). In guinea pigs, NO was continuously increased over the observed time period irrespective of the NOS activation. An imbalance in the equilibration of enzyme activity and increased NO production can be associated with cell damage (Darley-Usmar et. al., 1995).

There is a significant generation of oxidative species seen in xylene exposed skin in both species, which may reflect the damaging effects on the skin. Additionally, xylene-induced peroxidations of membrane phospholipids further strengthening the potential association of oxidative stress and skin damage. Corsini et al (1996 a, b) reported that oxidative radicals formed following irritant exposures can disturb the functional mitochondrial respiratory chain in the epidermis, and mediate the transcription factors activation, which could promote IL-1α expression. In the present study, the significant accumulation of cellular oxidative stress that documented within 1 hr xylene-post exposure correlated with IL-1α release. Increased intensity of oxidative species observed

in guinea pig than in rats in this study, results in severe histological damage including epithelial necrosis and cells infiltration. Recently, a phenol-induced oxidative radicals and cell death has been demonstrated in epidermal cells and fibroblasts (Shvedova et al., 2000; Newby et al., 2000). Phenol at high concentration induced severe oxidative stress with significant change of phospholipids and in turn leads to cytotoxicity (Goldman et al., 1999; Day et al., 1999). In the present study, generation of oxidative species could be another mechanism through which xylene induces skin damage.

SUMMARY

In the present study, the difference in the time course of biomarkers induction and severity of skin damage between rats and guinea pigs may be due to differences in the epidermal barrier function and metabolic factors that involved in the transformation of the xenobiotic (van de Sandt, et al., 1999). These rat and guinea pig models illustrate a spectrum of biological markers in xylene-induced acute skin irritation. The responses obtained from the guinea pig model seemed more sensitive to xylene-induced skin damage than the rat model. The rat model responded sufficiently to xylene, and it allowed us to rapidly evaluate the biomarkers efficiently with less inconvenience due to the availability of commercial products. Hence, we have decided that a rat model could be used in further studies testing the effects of xylene-induced irritation.

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